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The cell biology of glycosphingolipids

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Abstract

Glycosphingolipids, a family of heterogeneous lipids with biophysical properties conserved from fungi to mammals, are key components of cellular membranes. Because of their tightly packed backbone, they have the ability to associate with other sphingolipids and cholesterol to form microdomains called lipid rafts, with which a variety of proteins associate. These microdomains are thought to originate in the Golgi apparatus, where most sphingolipids are synthesized, and are enriched at the plasma membrane. They are involved in an increasing number of processes, including sorting of proteins by allowing selectivity in intracellular membrane transport. Apart from being involved in recognition and signaling on the cell surface, glycosphingolipids may fulfill unexpected roles on the cytosolic surface of cellular membranes.
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Keywords: Glycosphingolipids; Glycolipids; Translocators; Lipid rafts; Glycosyltransferases

1. Introduction

Eukaryotes (and a few bacteria) contain a heterogeneous class of lipids called glycosphingolipids (GSLs): these lipids are composed of a ceramide backbone and a sugar headgroup. The hydrophobic ceramide part, consisting of a sphingoid base and a fatty acid, is inserted in a cellular membrane, whereas the sugar headgroup mostly faces the non-cytosolic space. Knockout studies in mice have started to reveal the importance of GSLs in organism development. Importantly, a knockout in the enzyme responsible for the synthesis of glucosylceramide (GlcCer), producing mice without glucose-based GSLs, was found to be embryonically lethal [1]. The removal of GSLs is also of physiological importance, as shown by the serious pathologies induced when the degradation of GSLs is deficient [2], highlighting again the importance of the balance between their synthesis and degradation. At the cellular level, GSLs exert important signaling functions possibly in the form of glycosignaling domains [3,4]. In addition, cells use the capacity of GSLs to form ordered domains to create selectivity in membrane transport, important in the spatial organization of cells [5]. This review will focus on the synthesis of the main classes of mammalian GSLs, which are derived from the simple GSLs GlcCer and galactosylceramide (GalCer), and will discuss the sorting and transport of these GSLs, and their main functions in cells.

2. Diversity, synthesis and physical properties of glycosphingolipids

2.1. Heterogeneity of glycosphingolipids

GSLs exhibit a huge heterogeneity of structure, both in backbone and headgroup: more than 60 different sphingoid bases and more than 300 different oligosaccharide chains have been characterized so far, the combination of which creates thousands of different structures (Fig. 1). The sphingoid bases can vary in length, saturation, hydroxylation, and branching [6]. The main sphingoid base in mammals is sphingosine, or D-*erythro*-1,3-dihydroxy, 2-aminooctadec-4-ene or *trans*-4-sphingenine (d18:1). For example, in bovine kidney, sphingosine accounts for 80% of the total sphingoid bases [7]. Sphinganine, which corresponds to sphingosine without the *trans*-C4–C5 double bond, and phytosphingosine (C4-OH sphinganine), are also common sphingoid bases [8,9]. The fatty acid is amide-linked to the amino group of the sphingoid base. The fatty acid species are cell-type dependent, can vary in length, saturation and hy-

Abbreviations: CGalT, UDP-Gal:ceramide galactosyltransferase; CGlcT, UDP-Glc:ceramide glucosyltransferase; EGF, epidermal growth factor; ER, endoplasmic reticulum; Fuc, fucose; Gal, galactose; GalCer, galactosylceramide; Glc, glucose; GlcCer, glucosylceramide; GlcSph, glucosylsphingosine; GlcA, glucuronic acid; GPI, glycosylphosphatidylinositol; GSL, glycosphingolipid; MAM, mitochondria-associated membrane; Man, mannose; MDCK, Madin–Darby canine kidney; SM, sphingomyelin; PC, phosphatidylcholine

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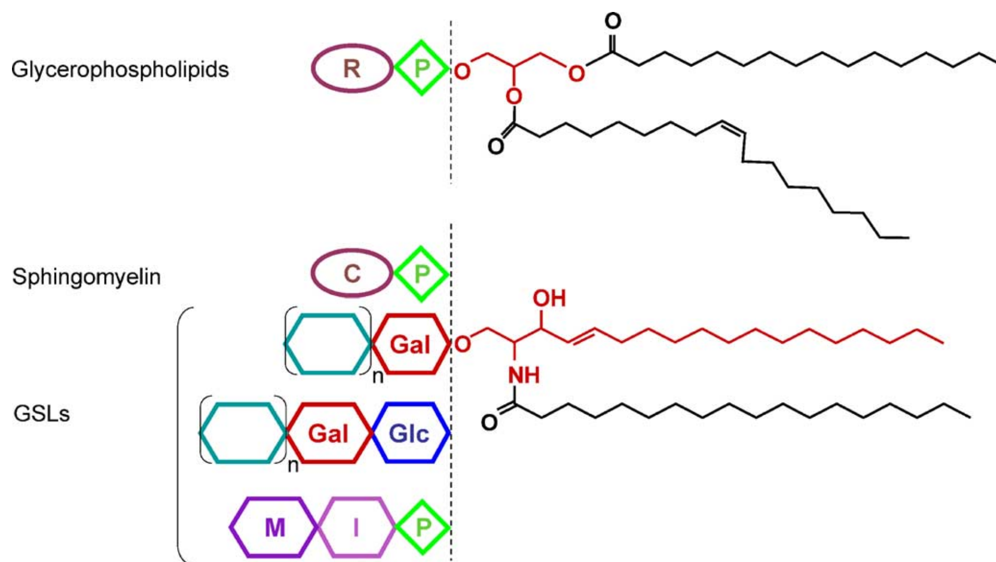

Fig. 1. Structure of phospho- and glycolipids. Glycerophospholipids contain a phosphorylated glycerol backbone (red), substituted by two fatty acids, and the headgroups (R) choline, ethanolamine, inositol, or serine yielding PC (phosphatidylcholine), PE, PI and PS, respectively. Sphingolipids contain a sphingoid base like sphingosine (red) with an amide-linked fatty acid. The common mammalian phosphosphingolipid is the choline-containing sphingomyelin (SM). The glycosphingolipids (GSLs) carry sugars on the ceramide backbone. In mammals, the two main classes of GSLs contain either Gal or Glc as the first sugar. GalCer can then be galactosylated or sulfated. GlcCer can be further glycosylated to LacCer (Galβ1-4GlcCer), which serves as a precursor for different series of GSLs. Fungi and plants, besides GlcCer and complex GSLs produce the SM analog inositolphosphoceramide, which can be elongated by mannose and/or glucuronic acid.

droxylation, but are mostly long (≥C16) and saturated in mammals [10], sometimes they are unsaturated at C15 or hydroxylated at C2. GSLs can be divided into two main classes based on the first sugar linked to the ceramide backbone, glucose (Glcβ1-) or galactose (Galβ1-). In more specific cases, other sugars have also been found linked to ceramide: Fucα1-Cer in human colon cancer cells [11], GlcACer in *Flaviobacterium* [12], Manβ1-Cer in several invertebrates [13]. The major GSLs in plants and fungi are based on inositolphosphoceramide, whereby mannose (α1–2, oρ α1–3) or glucuronic acid (α1–6) is transferred onto the inositol [14]. The common sphingoid base in these lipids is phytosphingosine, commonly C8 desaturated or, only in fungi, C9 methylated [15]. The fatty acids are very long (C26) and often 2-hydroxylated.

2.2. Synthesis of glycosphingolipids

The first step in GSL biosynthesis is the condensation of serine and palmitoyl-CoA to 3-ketosphinganine, the precursor for all sphingoid bases. Subsequent acylation produces ceramide [16,17]. In animals, the Δ4-*trans*-unsaturation or C4-hydroxylation of the sphingoid base most likely occur at this stage by a pair of newly identified desaturases DES1 and DES2 18 on the cytosolic side of the ER [18–20]. Ceramide can spontaneously cross the ER membrane and, in specialized cell types, be converted to GalCer. From the ER, ceramide can also follow the vesicular pathway to early Golgi compartments where it is converted to GlcCer. Alternatively, ceramide reaches the *trans*-Golgi via CERT, a newly identified ceramide transfer protein that is able to extract ceramide from the ER and deliver it to the Golgi after docking to phosphatidylinositol-4-phosphate [21–23]. This pathway may involve the membrane contacts between the ER and the *trans*-Golgi [24]. Synthesis of the phosphosphingolipid sphingomyelin (SM) by the newly identified SM synthase in the *trans*-Golgi [25] depends on this pathway for ceramide supply [21].

In mammals, GalCer derived GSLs are only present in specialized cells; they are the major lipids of the myelin sheath assembled around the axons of neuronal cells by oligodendrocytes and Schwann cells. In some animals, they are also present in epithelial cells of renal tubules and the gastrointestinal tract [5]. The transfer a Gal residue from UDP-Gal to ceramide occurs in the ER and is catalyzed by the UDP-Gal:ceramide galactosyltransferase or GalCer synthase CGalT [26–29]. CGalT is a class I integral membrane protein, belongs to the family of glucuronyltransferases, has an ER retention signal, and its active center is in the ER lumen [30]. It obtains its UDP-Gal substrate by retaining part of the Golgi UDP-Gal transporter in the ER [31]. On its way to the plasma membrane, GalCer passes the lumen of the Golgi apparatus. Here it can be sulfated to HO_3S-3GalCer (sulfatide). Especially in kidney epithelia, it can be galactosylated to Galα1-4GalCer. CGalT also produces animal Gal-diradylglycerol, which can be 3′-sulfated to seminolipid or, in plant chloroplasts, can be 6′-phosphorylated [32]. The important functions of the galactosyl lipids are illustrated by the physiological defects in the knockout mice described by Furukawa et al. in this issue.

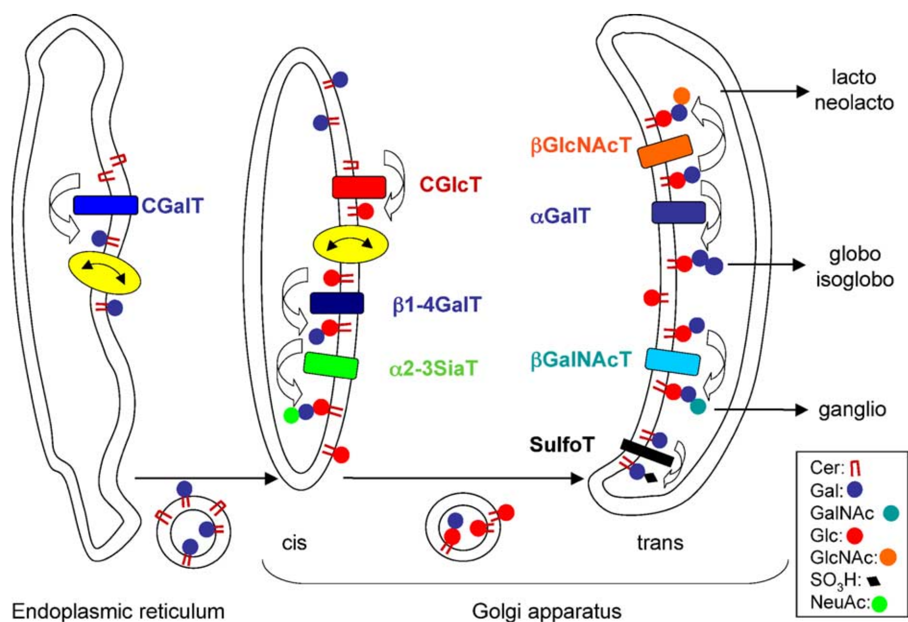

Fig. 2. Topology of mammalian GSL synthesis. Ceramide is produced in the endoplasmic reticulum, where the sphingoid base can be desaturated or hydroxylated. In specialized cells, ceramide can be converted to GalCer in the ER lumen by the Gal transferase CGalT, also called GalT-1 [158]. The glucosyltransferase CGlcT, active on the cytosolic side of the Golgi apparatus, synthesizes GlcCer which must be translocated to the Golgi lumen to be converted to LacCer by the β1,4-galactosyltransferase GalT-2 [158] also called Gal-T1 in a different nomenclature [159]. LacCer is the precursor of different series of GSLs, mostly only one in any specific cell type. GalCer and LacCer can be sulfated in the late Golgi.

In contrast to GalCer, GlcCer is present in most eukaryotic cells and a few bacteria and serves as the major precursor for complex GSLs. It is synthesized by the UDP-Glc:ceramide glucosyltransferase or GlcCer synthase CGlcT, which has been cloned from many organisms [33,34]. Sequence analysis suggests that CGlcT is a type III protein but, interestingly, it has no structure similarity with other proteins, including other glycosyltransferases. GlcCer synthesis occurs on the cytosolic side of Golgi membranes [35–37]. CGlcT activity has been detected not only in the early Golgi compartments, but also in another compartment, possibly a pre-Golgi compartment 36 or the late Golgi [37]. This observation could reflect the existence of two pools of GlcCer in cells, possibly having different functions.

Complex GSLs are made by the stepwise addition of individual sugars from their activated nucleotide precursors onto GlcCer. In mammals, the first reaction is the conversion of GlcCer to lactosylceramide (Galβ1-4GlcCer, or LacCer) by the LacCer synthase [38,39]. This enzyme, like all other glycosyl- and sufotransferases involved in the glycosylation of GSLs, acts in the Golgi lumen [40,41]. Several galactosyl-, *N*-acetylgalactosaminyl-, *N*-acetylglucosaminyl-, sialyl-, and fucosyltransferases can elongate the oligosaccharide chain of mammalian GSLs [42]. Some of these enzymes are involved in the formation of the oligosaccharide backbone, the structure of which defines the different series of GSLs (see Fig. 2 and Table 1). Other enzymes, like sialyltransferases and fucosyltransferases, are involved in the synthesis of the periphery of the oligosaccharide. Sialoglycosphingolipids are also called "gangliosides", and abbreviations have been assigned to them according to the number of sialic acids present and to their migration order in chromatography. The main abbreviations used are listed in Table 2 (Svennerholm system [43]). Except for ganglioside GM4, which corresponds to NeuAcα2-3GalCer, all gangliosides have LacCer as a precursor. Gangliosides are abundant in brain and nervous tissues. The carbohydrate chain of GSLs can also be sulfated by two different sulfotransferases: the Gal-3-*O*-sulfotransferase [44,45] in tissues that synthesize GalCer, mostly producing HSO_3-3GalCer but also HSO_3-3LacCer, a potential risk factor for type I diabetes mellitus, and a GlcA-3-*O*-sulfotransferase, responsible for the synthesis of the HNK-1 epitope (HSO_3-3GlcAβ1-3Galβ1-3/4GlcNAcβ1-3Galβ1-4GlcCer) observed in developing central nerve and cauda equina in peripheral nerve [46].

Table 1
Root names and structures (recommendations for the nomenclature of glycolipids, http://www.chem.qmul.ac.uk/iupac/misc/glylp.html [43])

Root	Symbol	Structure
Ganglio	Gg	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-Cer
Lacto	Lc	Galβ1-3GlcNAcβ1-4Galβ1-4Glcβ1-Cer
neolacto	nLc	Galβ1-4GlcNAcβ1-4Galβ1-4Glcβ1-Cer
Globo	Gb	GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-Cer
Isoglobo	iGb	GalNAcβ1-3Galα1-3Galβ1-4Glcβ1-Cer
Mollu	Mu	GlcNAcβ1-2Manα1-3Manβ1-4Glcβ1-Cer
Arthro	At	GalNAcβ1-4GlcNAcβ1-3Manβ1-4Glcβ1-Cer

Table 2
Some abbreviations for gangliosides in the Svennerholm system [43]

Abbreviation	Structure
GM4	NeuAcα2-3Galβ1-Cer
GM3	NeuAcα2-3Galβ1-4Glcβ1-Cer
GM2	GalNAcβ1-4 (NeuAcα2-3)Galβ1-4Glcβ1-Cer
GM1a	Galβ1-3GalNAcβ1-4 (NeuAcα2-3)Galβ1-4Glcβ1-Cer
GM1b	NeuAcα2-3Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-Cer
GD3	(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-Cer
GD2	GalNAcβ1-4 (NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-Cer
GD1a	NeuAcα2-3Galβ1-3GalNAcβ1-4 (NeuAcα2-3)Galβ1-4Glcβ1-Cer
GD1b	Galβ1-3GalNAcβ1-4 (NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-Cer
GT1a	NeuAcα2-8NeuAcα2-3Galβ1-3GalNAcβ1-4 (NeuAcα2-3)Galβ1-4Glcβ1-Cer
GT1b	NeuAcα2-3Galβ1-3GalNAcβ1-4 (NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-Cer
GT1c	Galβ1-3GalNAcβ1-4 (NeuAcα2-8NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-Cer

Which sugars are present in the oligosaccharide backbone and periphery depends on the glycosyltransferases present and active in the Golgi apparatus of the cell, which may vary depending on the cell type, the developmental stage, or disease stage [45,47,48]. For example, the presence of blood group ABH and of Lewis antigens will depend on the expression of certain glycosyltransferases (α1,3-*N*-acetylgalactosaminyltransferase, α1,3-galactosyltransferase, α1,2-fucosyltransferase for the ABH system, α1,2- and α1,3/4-fucosyltransferases for the Lewis system), which is genetically defined. The expression of β1,3-*N*-acetylglucosaminyltransferase, which is involved in the biosynthesis of glycolipids of the lacto- and neolacto-series, is highly regulated in mice and decreases after birth to undetectable levels in most cell types [49]. Dramatic changes in glycolipid glycosylation and metabolism have been described in many cancer cells. Most of the time, these modifications in glycosylation correspond to aberrant sialylation and/or fucosylation [50]. The specific tumor associated antigens formed can be involved in adhesion events, or trigger intracellular signaling cascades involved in the development of the disease [51].

Finally, in the yeast *Saccharomyces cerevisiae*, most of the genes involved in the biosynthesis of the inositolphosphosphingolipids have been identified [14,52–54]. Like inositolphosphoceramide synthesis, its mannosylation by the mannosyltransferases Csg1p and Csh1p occurs in the lumen of the medial Golgi [52,55].

GSLs are degraded in the lysosomes, where they are sequentially hydrolyzed from the non-reducing end by specific hydrolases. Interestingly, GlcCer can be degraded not only by a glucocerebrosidase in lysosomes, but also in the cytosol by a non-lysosomal glucocerebrosidase [56]. Defects in the degradation of GSLs (either in glycosidases or their activator proteins) result in GSL storage in lysosomes, the species stored depending on which enzyme is deficient, and often cause very serious pathologies. Treatment of these diseases include replacement of the enzyme, gene therapy and inhibition of synthesis [2].

2.3. Physical properties of GSLs

Biophysical differences between GSLs and glycerolipids underlie the special behavior of GSLs in membranes. In GSLs, the region between the polar headgroup and the hydrophobic backbone contains chemical groups that can function both as hydrogen bond donor and hydrogen bond acceptor, in contrast to the glycerolipids, which only have hydrogen bond accepting properties in that part of the molecule [57]. Additional hydrogen bonding can occur between the sugar headgroups of GSLs. Another striking difference between GSLs and most glycerolipids is the fact that the lipid chains are saturated over at least the first 15 carbons of both chains (without disturbance by the double bond at the sphingosine C4 which is in the *trans*-configuration). In combination with the higher hydrogen bonding, the saturated nature results in denser packing. This is measured as an increased melting temperature (T_m), which corresponds to the temperature above which a bilayer of a single lipid switches from a frozen state, the gel or solid-ordered (s_o) phase to a fluid state, the liquid-crystalline or liquid-disordered (l_d) phase. GSLs have a much higher T_m than glycerolipids. The structural differences allow sphingolipids to self-associate amongst glycerophospholipids in the plane of the membrane as a flexible hydrogen-bounded network. In addition, cholesterol, a rigid and flat cylindrical lipid, also interacts preferentially with sphingolipids via van der Waals interactions [58].

Studies in model membranes made of binary mixtures of lipids with different T_m have shown that phase separation can occur, resulting in the co-existence of a s_o and a l_d phase. Nevertheless, this type of phase separation is probably not of physiological significance in mammalian cells, since membranes that contain high amounts of lipids of high T_m (like the plasma membrane) also contain high amounts of cholesterol, and cholesterol at high concentrations abolishes the s_o/l_d phase transition [59]. Interestingly, in model membranes containing mixtures of a high T_m lipid and cholesterol, a fluid–fluid phase separation has been observed between the l_d and a liquid-ordered l_o phase [60]. In addition, phase-behavior experiments using three differ-

ent lipids of high T_m (di-saturated PC or a sphingolipid), low T_m (mono-unsaturated PC) and cholesterol [61–64] have shown that sterols have the ability to modulate the phase separation of lipids, depending on the concentration and the structure of the sterol used. Recently, de Almeida et al. have studied the co-existence of l_o, l_d and s_o phases in ternary mixtures of SM/mono-unsaturated PC and cholesterol. At a 1:1:1 ratio and 37 °C, it can be calculated that the l_o phase covered two-thirds of the surface, it was enriched in SM (1.5-fold) and cholesterol (four-fold) compared to the l_d phase, which in turn contained a four times higher PC concentration [65]. Partitioning between such domains of a protein with a glycosylphosphatidylinositol (GPI) anchor was affected by the presence of 1 mol% of the ganglioside GM1 [66].

The relevant question for cell biology is: do l_d/l_o phase separations occur in biological membranes and are they of relevance for GSL distribution and function? The structural differences between GSLs and glycerophospholipids that promote their phase separation are conserved from vertebrates to yeast [67]. Unfortunately, most evidence to date that domains enriched in GSLs exist in biological membranes is indirect [68]. Most convincing are probably the microscopical observations that GSLs are clustered on erythrocytes [69], that the gangliosides GM1 and GM3 were concentrated in domains [70,71] and that GM1 can be enriched in different domains than GM3 on the same cell [72]. However, most indications for the lipid domain association have been obtained by using the detergent-resistance criterion. A number of membrane components that had been predicted to be in these "lipid rafts" from other approaches turned out to remain associated with membrane remnants after extraction with cold detergent [73]. Although this technique has had great prospective value for how membrane signaling may work, especially at the immunological synapse [68], physical studies have suggested that there is no straightforward physical basis for why extraction at 4 °C would provide information concerning the situation at 37 °C [65,74,75]: the results may be correct but by coincidence [65], which calls for caution in the interpretation of such data.

In order to avoid detergent extraction, pulse EPR (electron paramagnetic resonance) spin-labeling methods and single-molecule optical techniques have been applied [66,76,77] to monitor the entry and exit of probe molecules in domains in model membranes or in living cells. The results have provided evidence for small/unstable rafts in unstimulated cells and for larger stabilized rafts induced by oligomerization of GPI-anchored proteins or ligand binding [68], in which some proteins preferentially partition. Interestingly, the size of the confining domain for a GPI-anchored protein is reduced when cells are treated with inhibitors of GSL synthesis, suggesting that GSLs contribute to the physical properties of microdomains [78]. In conclusion, many studies suggest that GSL can be heterogeneously organized on living cells, although there is no strong consensus yet on the size, shape and dynamics of lipid rafts.

3. Traffic of glycosphingolipids

3.1. Subcellular distribution of glycosphingolipids

As would be expected from the location of many of their functions, GSLs have been found at the outside of the plasma membrane, although they usually constitute a minor fraction of the total plasma membrane lipids [79]. One exception is the apical plasma membrane of epithelial cells of the intestinal and urinary tracts, where GSLs are found at particularly high concentrations of 30–40 mol% of total lipid [80–83]. In comparison with the basolateral membrane of these cells, GSLs are almost two to four-fold enriched in the apical membrane [80–84]. Because the tight junction forms a lipid diffusion barrier between these two membrane domains, but exclusively in the non-cytosolic leaflet, that is where the differences in lipid composition must exist. If all GSLs were in the non-cytosolic leaflet and cholesterol would be distributed evenly over both leaflets, the apical surface of these cells would be completely covered by GSLs! High concentrations of galactosyl-GSLs also exist in myelin, while neuronal plasma membranes are rich in gangliosides. Intracellularly, GSLs are found in the vacuoles of the exocytic and endocytic pathways, with the exception of the ER that is low in GSL content [85,86]. As for mitochondria and peroxisomes, in general, no or very low levels of GSLs were found in these organelles. Indeed, Forssman glycolipid, a five-sugar GSL of the globo-series, was found at the plasma membrane, in endocytic compartments, to a low extent in the ER but excluded from mitochondria and peroxisomes [86]. However, a highly increased content of the ganglioside GD1b was reported for mitochondria of malignant hepatoma [87], and GD3 was found in mitochondria in ceramide-induced apoptotic cells [88]. Still, it has generally not been excluded that the GSLs actually resided in the mitochondria-associated membrane, MAM, an ER subcompartment that closely associates and copurifies with mitochondria. The MAM has been reported to contain the enzymes of early GSL synthesis [89]. The various GSLs apparently display different distributions over the various intracellular membranes [85,90]. Notably, while complex GSLs were found enriched on the plasma membrane, more of the GlcCer was localized to intracellular membranes [91].

3.2. Glycosphingolipid transport and sorting

As most GSLs (and SM) are synthesized at the lumenal, non-cytoplasmic side of the Golgi, and reside on the plasma membrane surface and on the lumenal side of the endocytic organelles, the main transport mechanism must be the vesicular transport along the exocytic and endocytic recycling pathways (Fig. 3). Indeed, it was shown for GM3 and for SM that they can only be transported from the Golgi to the plasma membrane via vesicular transport [92,93]. Also the recycling of GSLs and SM via the endocytic vesicular transport pathways has been described in great detail [94].

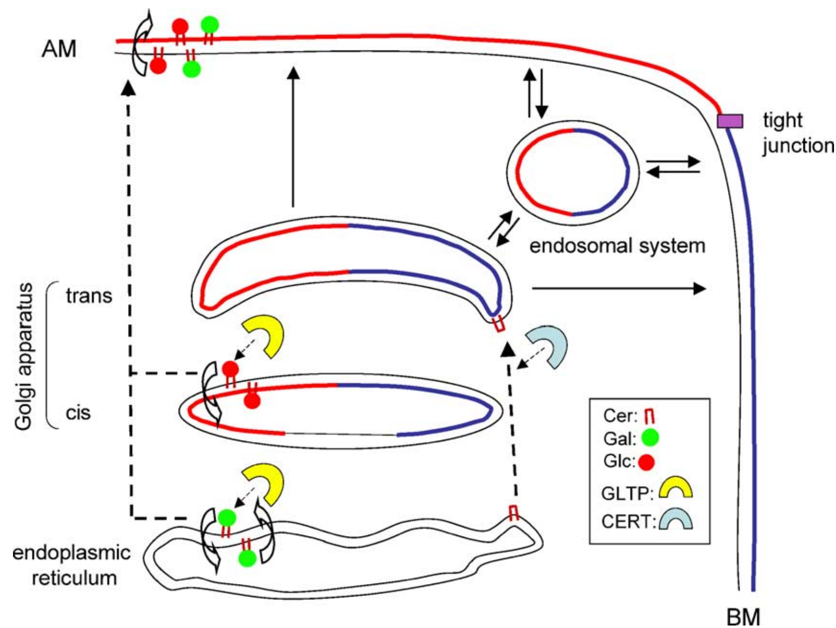

Fig. 3. Intracellular transport of GSLs. Ceramide can be transported from the ER to the *cis*-Golgi by vesicles. Alternatively, it is transported by the transfer protein CERT to the *trans*-Golgi [21], most likely via ER-Golgi contact sites [22]. GalCer is able to translocate freely across the ER membrane [160], and is available for the glycolipid transfer protein GLTP, as is GlcCer on the cytosolic surface of the Golgi [105]. GlcCer can flip across Golgi membranes spontaneously [107,160], or by the same ABC transporter as in the plasma membrane, most likely MDR1 [109–111]. In the lumenal leaflet of the Golgi lipids destined for the apical surface (red) are sorted laterally from basolateral lipids (blue) and from lipids in the retrograde direction (black).

Since all vesicular transport pathways are directly or indirectly bidirectional, a major question has been how the cell maintains the different lipid compositions of its organelles. For example, how are newly synthesized GSLs preferentially transported to the plasma membrane and not to the ER, how are they preferentially incorporated into the apical pathway and into a specific endocytic pathway? Experiments on epithelial cells have led to one unifying hypothesis for the mechanism of GSL sorting: the lipid raft model [5,95]. In this model which has been refined over the years by the contributions of many, sphingolipids in general and GSLs in particular form l_o domains in the non-cytosolic leaflets of the Golgi, plasma membrane and endosomes. Because phase boundaries are energetically unstable, rafts will have a tendency to minimize the length of the boundary by budding into a vesicle [96,97] (Fig. 4). These domains should incorporate proteins that can impose transport specificity, and, for a start, certain SNARE molecules involved in docking and fusion have now been reported to preferentially partition into lipid rafts [98–101]. It has been argued that the occurrence of a single type of lipid raft would be insufficient to explain the lipid sorting in the *trans*-Golgi network of epithelial cells, where lipids to be transported back to the ER, unsaturated glycerophospholipids, would have to be segregated from basolateral lipids, SM and cholesterol, and from apical lipids, GSLs and cholesterol [92,93]. The work on GSL sorting at the plasma membrane between pathways towards the Golgi and to the late endosomes clearly shows that GSL sorting can be regulated. The role of coat proteins like caveolin in the process is now being unraveled [102,103].

The fact that GlcCer is synthesized on the cytosolic surface of the Golgi implies that it can in principle follow three transport pathways [104]: (1) If it moves into budding transport vesicles it can reach the cytosolic surface of all membranes of the exocytic/endocytic membrane system. (2) It may be extracted from the membrane by a transfer protein and transferred to the cytosolic surface of other organelles, e.g. the mitochondria or peroxisomes. A glycolipid transfer protein has been identified [105], and cell fractionation has suggested that vesicle independent transport of GlcCer between Golgi and plasma membrane does indeed occur [106].

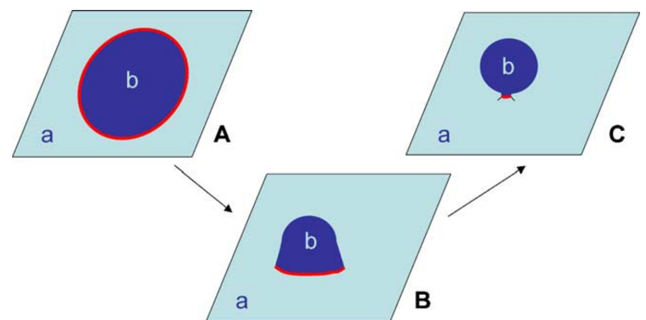

Fig. 4. Behavior of co-existing fluid lipid phases. In a membrane containing co-existing fluid phases (a) and (b), with the (b) phase surrounded by the (a) phase (panel A), budding of the (b) domain will occur as soon as its size exceeds a certain threshold value (panel B), provided the tension within the membrane is sufficiently small. Very often, the final shape of (b) is a complete bud connected to (a) by a very thin neck (panel C). This model by Lipowsky and Dimova [97] was recently proven experimentally to be correct [96].

The function of this process is presently unclear. (3) GlcCer may flip to the lumenal leaflet of the Golgi membrane, where it becomes available for the LacCer synthase or where it can enter the lumenal aspect of transport vesicles. Evidence for fast, energy-independent GlcCer translocation across the Golgi membrane has been presented [107]. In contrast, evidence has been presented to suggest that GlcCer is a substrate for the ABC-transporters MDR1 P-glycoprotein and MRP1 [108]. So, the possibility exists that GlcCer is removed from the cytosolic surface of the Golgi [109,110] or the plasma membrane [111], and that its presence at the cytosolic surface reflects a hitherto unknown function. Actually, after synthesis in the ER lumen also GalCer is expected to have access to the cytosolic leaflet, and it may therefore have the same fate and function as GlcCer.

It is fully unclear how gangliosides like GD3 and Gd1b reach the mitochondrion [88], because even if these lipids made it back to the ER lumen via the retrograde route, the experimental evidence suggests that complex GSLs are not able to translocate to the cytosolic surface [107]. The fact that Forssman glycolipid was not found in mitochondria may suggest that there may be a selective transport process for the gangliosides.

4. Functions

4.1. Lessons from knockout mice

The importance of GlcCer-derived GSLs became obvious when the observation was made that embryos from a mouse with a knockout for this enzyme die at 7.5 days [1], which shows that GSL synthesis is critical for embryonic development and for differentiation of certain tissues. Interestingly, a melanoma cell line GM95 that does not have the CGlcT lives [112]. Nevertheless, these cells have a defect in melanogenesis, linked to a sorting defect of melanosomal proteins [113]. This role of GSLs in protein sorting will be developed below. Knockouts for the ceramide glucosyltransferase have also been made in *P. pastoris* and *Candida albicans*: they resulted in a complete loss of GlcCer, but this had no essential effect on cell growth [34].

Other mouse models lacking enzymes involved in the biosynthesis of GSLs have been made. Mutant mice lacking the GM3 synthase (α2,3-sialyltransferase) appear rather normal but exhibit an enhanced insulin sensitivity, probably due to an increased phosphorylation of the insulin receptor [114]. KO mice for the GM2/GD2 synthase (a β1,4-GalNAc transferase) are also viable, but exhibit defects in the nervous system, the maintenance and repair of nervous tissues, the differentiation of spermatocytes, and the regulation of the IL2 receptor complex [115–118]. Double KO mice lacking the GM2/GD2 and GD3 synthases (β1,4-GalNAc transferase and α2,8-sialyltransferase, respectively) develop skin injury more rapidly, showing a role of higher GSLs in maintaining skin integrity [119].

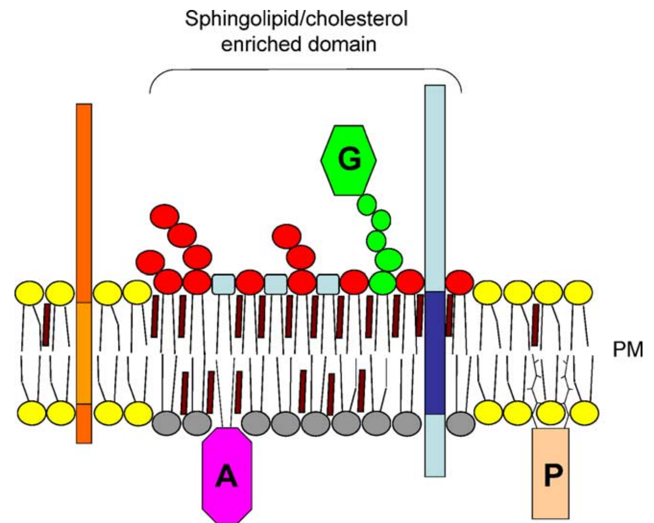

Fig. 5. Schematic model of a sphingolipid/cholesterol raft. GSLs have a tendency to form a hydrogen-bonded network with SM and cholesterol in a phospholipid environment, forming rafts on the non-cytosolic side of the plasma membrane. GPI-anchored proteins (G) are enriched in these rafts. The composition of the inner leaflet of GSL-enriched microdomains is unknown, but is probably special since only acylated proteins (A) are thought to associate with the cytosolic leaflet of rafts, whereas prenylated proteins (P) are excluded [161]. Proteins that travel to the plasma membrane have in general longer transmembrane domains than Golgi resident proteins [152], and therefore may associate with GSL-enriched microdomains that are thicker than a typical phospholipid membrane [162].

Mice with a knockout in the CGalT are alive and lack GalCer and sulfatide [120,121]. These mice display defects in the nervous system. The male were infertile because spermatogenesis was blocked before the first meiotic division; this was due to the absence of monogalactosylalkylacylglycerol and seminolipid (HO_3S-3Galβ1-alkylacylglycerol) in testis [122]. These data show a critical role for sulfoglycolipids in myelin function and spermatogenesis. Altogether, the observations made in mutant mice reflect the crucial role of GlcCer in embryonic development and differentiation, and the involvement of more complex GSLs in a number of important processes, especially in the nervous system.

4.2. Glycosphingolipids are part of ordered sphingolipid domains in cellular membranes and key molecules in recognition and signaling

As mentioned above, because of their tightly packed backbone GSLs have the property to interact preferentially with themselves and with SM and cholesterol in a phospholipid environment, therefore forming rafts [123,124] (Fig. 5). Raft formation is thought to be the basis for many of the properties of GSLs. They are thought to be enriched not only in specific proteins in their lumenal leaflet [125,126], but also in peripheral proteins carrying myristoyl and palmitoyl chains in their cytoplasmic leaflet, such as src-family kinases which are related to signal transduction [3,4]. The notion of the glycosynapse has emerged, defined as a microdomain at

the cell surface involved in glycosylation-dependent adhesion/recognition and signaling [127].

A number of studies have shown that GSLs are involved in many aspects of cell signaling. First, the carbohydrate part of GSLs itself is involved in a number a recognition processes: some saccharide determinants present in the carbohydrate chains of GSLs, generally at the periphery, can correspond to blood group antigens (ABH, P, Lewis, Ii systems), to development-associated antigens, or to abnormal disease-associated antigens. These epitopes are involved in several recognition processes, like cell–cell interaction, cell-substratum interaction, cell–pathogen interaction [128,129]. Interactions can occur between GSL and lectin-like proteins [130,131], or directly between two GSLs [132,133]. Because they can interact with other proteins or sugars, GSLs are targets for many pathogens that use GSL-enriched domains as platforms to enter the cells: these pathogens can be viruses (for example, influenza virus [134], HIV [135], rotavirus [136]), bacteria (*Helicobacter pylori* [137], *Neisseria gonorrhoeae* [138], *Escherichia coli* [139]) and several toxins (Cholera toxin, Shiga toxin/verotoxin, *Clostridium botulinum* toxin) [140–142] can also directly bind GSLs. Interaction between GSLs and their interaction partner can subsequently trigger a variety of events, like entry of the pathogen in the cell, adhesion, growth, differentiation, migration, and apoptosis.

Second, it is thought that GSLs present in microdomains are involved in the modulation of signal transduction: they can interact with key transmembrane receptors that will trigger intracellular signaling cascades, resulting in regulation of cell proliferation and/or differentiation. Two major transmembrane signaling systems operating in the majority of eukaryotic cells, the growth factor receptor-associated protein kinases and PKC, have been shown to be modulated by gangliosides or their degradation products. For example, GM3 would be involved in the regulation of the epidermal growth factor receptor signal transduction, by preventing the dimerization of the receptor and therefore inhibiting the receptor kinase [143]. Similarly, tyrosine phosphorylation of the platelet-derived growth factor receptor is inhibited by GM1 and other gangliosides [144]. PKC activity is inhibited by several glycolipids [145], including GD1b, GT1b, GM3. GSLs have also been shown to modulate integrin function [146].

Finally, GSL synthesis intermediates or metabolites, i.e. ceramide, sphingosine, sphingosine-1P, lysogangliosides, can lead to inhibition or activation of many intracellular processes and are known as key mediators in apoptosis, proliferation, and stress response [147,148].

4.3. Glycosphingolipids and protein sorting

GSL-enriched domains are thought to originate in the Golgi apparatus and it has been hypothesized that polarized epithelial cells use these domains as sorting platforms for the apical delivery of plasma membrane proteins [5]. Raft-association and subsequent apical sorting has been described for some transmembrane proteins and for GPI-anchored proteins [124]. Later, it appeared that rafts also play a role in sorting processes not only in polarized epithelial cells, but also in many other cell types. Notably, in yeast *S. cerevisiae*, lipid rafts are involved in the biosynthetic delivery of proteins to the plasma membrane [149–151]. One idea is that sphingolipid/cholesterol rafts are thicker than a typical phospholipid/cholesterol membrane [152] and that plasma membrane proteins recognize these via their transmembrane domains which are longer than those of Golgi proteins.

How important are GSLs in raft function? It has been shown that GSL deficiency affects the formation of functional microdomains in lung carcinoma cells [153]. Moreover, the addition of GM1 to CHO-K1 cells modifies the distribution of GPI-anchored proteins in the plasma membrane, showing an effect in the organization of microdomains [154], and the addition of gangliosides has been shown to displace GPI-anchored proteins from microdomains in MDCK cells [155]. In agreement, the reduction of GSL levels in rafts affects the expression and function of GPI-anchored proteins, but does not impair signal transduction via the T cell receptor [156]. Nevertheless, it was also shown that GSLs are not essential for the formation of detergent-resistant domains and GPI-anchored protein sorting in melanoma cells, probably because the mutant cell line compensates the GSL defect by increasing SM synthesis [157]. In yeast *S. cerevisiae*, the absence of mannosylated GSLs does not modify protein sorting in the late Golgi [52]. These results suggest that GSLs are not essential for the formation of detergent-resistant domains, but could be involved in more specific functions fulfilled by these domains.

In addition, sorting functions of GSLs are not restricted to plasma membrane proteins, but also occur at other locations. In the glycolipid-deficient melanoma cell line GM95, we have shown the importance of GSLs in the sorting of two melanosomal proteins, tyrosinase and tyrosinase-related protein 1 (TRP1), which results in a defect in melanosome biogenesis and pigmentation [113]. Tyrosinase was retained in the perinuclear region, whereas TRP1 could still reach the melanosomes but was misrouted via the cell surface. Sorting of melanomal enzymes and pigmentation could be restored when glycolipid-deficient cells were transfected with the CGlcT, or incubated with glucosylsphingosine (GlcSph). It is still not clear which GSL is involved in melanosomal protein sorting: it could be GlcSph itself, GlcCer synthesized by the cells when GlcSph is added, or a derivative or degradation product. The molecular mechanism of how GSLs are involved in the sorting of melanosomal proteins remains to be determined. It is tempting to speculate that GlcCer (or a derivative) on the cytosolic side of membranes could be required for the action of cytosolic proteins involved in the sorting of melanosomal enzymes. Alternatively, GlcCer could be part of domains necessary for the correct sorting of these enzymes.

5. Conclusion

In conclusion, the differential distribution and the specific pattern of higher GSLs in various tissues strongly suggests that they play important and specific functions in these tissues, and this has been confirmed by the observed pathological defects in knockout mice lacking specific glycosyltransferases. At the cellular level, the organizing potential of glycosphingolipids may play important roles in the lateral build-up of membranes. This appears to provide specificity in the sorting of proteins, notably proteins destined for the (apical) plasma membrane but also for melanosomes in pigmented cells. In addition, the self-organizing principles of glycosphingolipids in combination with their structural specificity have been proposed to fulfill crucial roles in signaling events at the cell surface. Unfortunately, these properties are being abused by a wide range of pathogens in invading cells. Finally, some glycosphingolipids occur on the cytosolic surface of cellular membranes. Amazingly, their fate and function is unknown which illustrates that we solved only part of the puzzle posed by these sphinx-like lipids.

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